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BIPHASIC PARTICULATE MEDIA FOR THE CULTURE OF FILTER-FEEDERS [1, 2]

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The principal conduit of nutrients between the primary producers and higher trophic levels in aquatic ecosystems is the micro-crustaceans. These herbivores which feed in nature on phytoplankton plus bacteria and fine detritus are, in turn, preyed on by various carnivores, such as small fish. While the trophic role of filter-feeding crustaceans in aquatic food chains has been extensively documented, little is known concerning their specific nutritional requirements. One reason for this deficiency has been the lack of artificial media which meet their specialized requirements as phagotrophs.

A new type of media in which nutrients are supplied as both particles and solutes (biphasic particulate media) has led to the establishment of a number of nutrient requirements for two of these crustaceans. The first chemically defined medium of this type allowed good survival and rapid growth from newborn to adult stages of the amphigonic race of the brine shrimp, *Artemia salina*, but the same medium supported growth only to juveniles for the parthenogenetic race (Provasoli and D'Agostino, 1969). A freshwater version of this medium for *Daphnia magna* gave similar results; growth to adult with only occasional progeny (Provasoli, Conklin and D'Agostino, 1970). While formulation of media supporting growth to adult stages is essential in defining cultural conditions and the major nutritional requirements, the lack of fertility of the animals indicated that these media were still nutritionally incomplete.

The missing fertility factors in filter-feeding crustaceans were studied, using the water flea *Moina macrocopa americana* which is viviparous, parthenogenetic, and has a much shorter life cycle. *Moina* was eventually grown for >200 germ-free consecutive generations on three artificial media—one of which is almost defined. This report describes the compounding of nutrient particles and discusses the possibility of using similar media to satisfy the phagotrophic requirements of other filter-feeding invertebrates.

[1] It has long been the policy of THE BIOLOGICAL BULLETIN not to accept methodological papers "which describe only a new technique or method" without extensive experimental results resulting from its use. In view of the difficulties encountered in earlier attempts at the axenic culture of filter-feeders, and the importance of these techniques to future studies in the physiology and productivity of a variety of aquatic invertebrates, it seemed appropriate to make an exception in this case—*Editor*.

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Materials and Methods

The original culture of *M. macrocopa americana* was obtained from Dr. James Murphy of the Rockefeller University. Following his suggestion (Murphy, 1970), monoxenic cultures were maintained using the algal species, *Chlamydomonas reinhardii,* until an adequate artificial medium (E medium) was developed. Early testing of artificial media was complicated by the necessity of eliminating the algal cells. This was done by 5–10 consecutive transfers of several animals in sterile media containing starch particles. Ingestion of the particles cleared the gut of algal cells which were eliminated from the medium by the repeated dilutions. Once the E medium (supplemented with lipid-rich particles containing serum, egg yolk and

Table I

Artificial media

E. medium: basal medium 98 ml + 2 ml trigel particles + 0.2 ml egg particles; pH 7.6–7.8.

FP medium: basal medium 97 ml + 2 ml trigel particles + 1 ml FP particles; pH 7.6–7.8.

F1 medium: basal medium 97 ml + 2 ml SA gel particles + 1 ml FV particles; pH 7.6–7.8.

Particles

Trigel particles: 2 ml supply 15 mg egg albumin + 10 mg rice starch + 5 mg dry beef serum.

Egg particles: 0.2 ml supply 10 mg egg yolk + 2 mg vitamin E (type II, Sigma Co.) + 0.5 mg calciferol.

FP particles: 1 ml supplies 4.5 mg albumin fraction V + 3 mg vitamin E (type II) + 1.5 mg egg lecithin + 0.75 mg calciferol.

SA gel particles: 2 ml supply 15 mg egg albumin + 10 mg rice starch.

FV particles: 1 ml supplies 6 mg albumin fraction V + 1.5 mg egg lecithin + 1 mg BHT (butylated hydroxytoluene) + 1 mg calciferol + 0.5 mg β-carotene + 2 mg *dl*-α tocopherol + 1 mg palmitic acid + 0.5 mg oleic acid + 1 mg linoleic acid + 1.5 mg linolenic acid.

Common basal medium (per cent w or v/v)

KCl, 3 mg; $MgSO_4 \cdot 7\ H_2O$, 4 mg; Ca (as Cl^-), 2 mg; K_3PO_4, 2 mg; $Na_2SiO_3 \cdot 9\ H_2O$, 2 mg; metal mix PII, 1 ml (1 ml contains Na_2EDTA, 1 mg; Fe, 0.01 mg; B, 0.2 mg; Mn, 0.04 mg; Zn, 0.005 mg; Co, 0.001 mg); Fe (as $(NH_4)_2$ H citrate), 0.05 mg; glycylglycine, 50 mg, pH 8.0 [TRIS buffer (Sigma Co.) is toxic for *Artemia, Daphnia* and *Moina* at 50 mg%. TES buffer (Sigma Co.) is nontoxic at 100 mg% for *Moina*]; nucleic acid mix V, 2 ml (1 ml contains adenylic acid, 20 mg; guanylic acid, 10 mg; cytidylic acid, 10 mg; thymidine, 10 mg; dissolve in alkali, adjust to pH 8.0); DF 2, 1 ml (1 ml contains Tween 60, 2 mg; Tween 80, 2 mg; rutin, 0.5 mg; oxbile extract (Nutritional Biochem Co.), 1 mg; disperse and emulsify components; adjust to pH 8.0); Cholesterol, 0.6 mg (dissolved in 95% ethanol, squirted into boiling water, ethanol boiled off; forms fine crystalline precipitate); amino acids mix III, 1 ml (1 ml contains L-isoleucine, 10 mg; L-lysine HCl, L-glutamic acid, L-histidine base, L-threonine, L-methionine, L-leucine, L-valine, L-proline, 1 mg each; L-arginine base, L-tyrosine, L-serine, glycine, L-tryptophane, 0.5 mg each); vitamin mix M1B, 1 ml (1 ml contains thiamine HCl, 0.5 mg; nicotinamide, 1.5 mg; pyridoxine HCl, 0.2 mg; biotin, 0.06 mg; putrescine·2 HCl, 0.1 mg; Vitamin B_{12}, 0.002 mg; choline H_2 citrate, 0.2 mg; riboflavin, 0.2 mg; folic acid, 0.1 mg; Ca pantothenate, 4 mg); liver infusion L 25 (Oxoid, Flow Labs, Rockville, Md.), 70 mg (does not dissolve completely; upon autoclaving in medium forms a brown precipitate essential for growth). Adjust pH of basal medium to pH 7.6–7.8.

vitamins D_2 and E; Table I) was developed, it was used both as the maintenance medium and the control medium during further work on substitution of serum and egg yolk with more chemically defined particles. Transfer techniques used for the bacteria-free *Moina* studies were essentially those developed for *Artemia* nauplii (Provasoli, Shiraishi and Lance, 1959).

The general form of the media is presented in Table I. This type of biphasic media is similar to those developed for culturing *Artemia*. The liquid phase contains salts and trace metals, pH and metal buffers, amino acids, nucleic acids and a mixture of water-soluble vitamins. The solid phase is a slurry of fine (up to 30 μm) particles of proteins, carbohydrates, and lipids. The addition of lipid-rich particles proved necessary for continuous generations of *Moina*.

Particle preparation

SA gel. Dissolve completely 750 mg of egg albumin (2X cryst., Sigma Chem. Co.) in 30 ml H_2O before adding 500 mg of rice starch. The mixture is then homogenized in a Virtis homogenizer model "23" (container #16–117) for a few minutes using two straight blades at right angles (Virtis blade #2–16–108). The mixture is autoclaved (20 min at 20 lb), cooled, homogenized for another 5 min at medium to high speed and reautoclaved. Following a final homogenization, the suspension is diluted to 100 ml with H_2O resulting in a fine, milky-white liquid. Autoclaving the gel twice prevents reaggregation of the particles during storage and also during the autoclaving of the complete medium.

Trigel. Water is added drop-wise to 250 mg of dried beef serum, avoiding lumps which would stick to the container wall, until the serum is completely dissolved. The mixture is then brought to 30 ml with H_2O. Then 750 mg albumin and 500 mg rice starch are added, and the mixture is homogenized and autoclaved following the procedure detailed for the SA gel. The final appearance of the trigel is a fine light-brown suspension.

Egg particles. A fresh egg yolk, free of albumin, is transferred without breaking to a 30 ml beaker. The membrane is penetrated with a 5 ml pipette, and the yolk material sucked up. Three ml of yolk is allowed to flow from the pipette into a test tube (16 × 75 mm). Free flow insures more repeatability than blowing out since it avoids differing amounts of yolk coating the pipette wall. Add 150 mg of ergocalciferol to 0.6 ml of a tocopherol concentrate (α-tocopherol type II, Sigma Chem. Co.) and triturate with a glass rod until completely dissolved. After addition of 10 ml of H_2O, the mixture is emulsified on the "Vortex Genie" (Scientific Industries Inc., Queens Village, New York) at top speed. The mixture is transferred into a Virtis container 16–117, rinsing the test tube twice with 10 ml of H_2O each time and emulsified further with 3 min of homogenization with the double blades. The emulsion is heated in a water bath on a hot plate with constant stirring until coagulated in large flocs. The egg mixture is then put through two cycles of autoclaving, cooling, addition of 5 ml H_2O, and homogenization for 3 min. Finally, the mixture is diluted to 60 ml with H_2O. The resulting light yellow suspension is stored in a glass-stoppered bottle, flushed with N_2, and refrigerated. Even though autoclaved twice, the egg particles tend to aggregate on storage and must be thoroughly agitated before use.

FV particles. A more defined mixture of lipids was specifically tailored to the needs of *Moina* and replaced the serum and egg yolk supplements of the E medium. To compensate for the emulsifying properties of the egg yolk, egg lecithin is used. Add 75 mg of egg lecithin and 300 mg of albumin (Fraction V, Sigma Chem. Co.) to 25 ml of H_2O in a Virtis flask (16–115). This flask has an enlarged bottom with small fluting and a side-arm capped with a small rubber plug on the top of the enlarged bottom. The lipid solution is prepared separately in a short test tube. The dry solids are added first, in the following order: butylated hydroxytoluene (BHT), 100 mg; ergocalciferol, 100 mg; β-carotene, 30 mg; and palmitic acid, 100 mg. Then in order: dl-α-tocopherol, 0.2 ml; linolenic acid, 0.15 ml; linoleic acid, 0.1 ml; oleic acid, 0.05 ml; and 1.5 ml of acetone. Stirring with a glass rod and use of the "Vortex Genie" helps to dissolve the mixture completely. One ml of the lipid mixture is drawn into a small hypodermic syringe with a thin needle. The albumin and lecithin are homogenized thoroughly for 2–3 min at top speed (with the 2 straight blades) before the 1 ml of lipid mixture is slowly squirted into the Virtis container through the rubber cap covering the side-arm. Homogenization is continued for 8 min at top speed, followed by autoclaving and cooling. The appearance after autoclaving is not uniform: a thin skin of coagulated material overlaps the liquid containing a flocculent mass. The skin and the coagulum are mixed and resuspended with a glass rod, then homogenized for 5 min. The above procedure of autoclaving and homogenization for 5 min is repeated once more and the final volume brought to 100 ml. The final appearance is a brownish-red suspension of fine particles.

Carotene is difficult to dissolve and is replaceable with 0.03 ml retinol palmitate (Type IV, Sigma Chem. Co) resulting in a more homogeneous initial coagulum. Increasing the fat-binding albumin fraction V (>600 mg) inhibits the growth of *Moina*. However, we found recently that when egg albumin, which can be used in higher concentrations, is substituted for fraction V, the resulting particles are again more homogeneous. Initially 300 mg of egg albumin plus 75 mg of lecithin are homogenized together in 25 ml H_2O. After adding the fat solution to the mixture, it is homogenized for 5 min at top speed, then an additional 500 mg of egg albumin is added; followed with another 5 min homogenization. The emulsion from the Virtis container is transferred to a 600 ml beaker and coagulated in a boiling water bath with continuous stirring. Following this rapid coagulation, the lipid particle mixture is autoclaved and homogenized twice as outlined above and brought to 100 ml. All the lipid particle mixes are stored refrigerated in glass stoppered bottles which have been flushed with N_2.

FP particles. Another lipid particle was also successful in replacing the egg particle (medium FP, Table I, Conklin and Provasoli, 1977). Dissolve 225 mg of albumin fraction V in 20 ml H_2O, add 75 mg of egg lecithin in a Virtis flask with side arm; homogenize for 3 min at top speed. Then add, as above, 1 ml lipid mixture [0.15 ml α-tocopherol type II (Sigma Chem. Co.) + 37.5 mg ergocalciferol dissolved in 1 ml acetone] through the side arm and homogenize for 8 additional minutes; follow as for FV particles with 2 cycles of autoclaving, cooling, homogenizing and bring to 50 ml. The simpler FP medium may be useful for other filter feeders.

While the proportions in the medium of the SA gel, trigel, and the FV and FP particles may be varied to suit other filter feeders, modifications in the composition of the gels and lipid particles should not exceed the limited binding power of the albumin. To insure a good coagulation and protein binding and to avoid separation of the lipids, it is necessary to use a small amount of H_2O (20–30 ml) in the initial mixture that is homogenized and coagulated for the first time by heat or autoclaving. The particles thus produced are stabilized by the second autoclaving and after the final homogenization can be dispersed in a large volume of H_2O (50–100 ml or more) without changing their physical properties.

Results

The media are biphasic. The mineral base [minerals, trace metal mix, and glycylglycine (at pH 7.8)] was a modification of the medium formulated for *Daphnia magna* (D'Agostino and Provasoli, 1970) which proved satisfactory for rearing this cladoceran in dixenic culture on *Chlamydomonas reinhardii* and *Scenedesmus obliquus*.

Assuming that essential nutrients for *Artemia* might also be required by *Moina*, various combinations of amino acid, nucleic acid and vitamin mixtures were used, and various quantities and ratios of starch and protein were co-gelled into fine homogeneous particles. A striking difference was seen in protein : starch ratios. Specimens of *Moina*, as well as those of *Daphnia*, seem to prefer a more even ratio of protein : starch (P : S = 1 : 0.5–2.0) in contrast to *Artemia* which needs a high starch ratio (P : S = 1 : 5). On the contrary, the requirements for most water soluble nutrients were similar although adjustments in concentrations were necessary. Media at this stage did not support consecutive generations for *Moina macrocopa*. Some adults were produced but the sparse progeny did not reach adulthood.

Failure of satisfactory viability presumably was due to lipid deficiencies : many insects need several fatty acids and some require tocopherol for fecundity and all need sterols (Dadd, 1973). *M. rectirostris* produced males, females and ephippial eggs in bacterized cultures fed defatted yeast supplemented with olive oil and ergosterol (von Dehn, 1955) ; fertility of *Daphnia magna* under similar conditions was thought to be restored by vitamin E (Viehoever and Cohen, 1938).

Early attempts to supply lipids as emulsions did not prove very useful. Efforts were then directed toward producing lipid-rich solid particles. A particle made up of starch, protein and serum (trigel; Table I) permitted one or two more generations. Additions of ergocalciferol and the vitamin E concentrate in an egg yolk carrier resulted in a repeatable preparation of highly nutritious particles. The Sigma Chemical Co. "Type II" α-tocopherol, an equal-part mixture of α-tocopherol and a vegetable oil, supplied a convenient array of fatty acids and an antioxidant. Coagulated egg yolk, added primarily as a carrier for the vitamin E oil, presumably also supplied a number of nutrients; however, the egg yolk alone was poor or inhibitory. In this lipid-rich medium, *Daphnia magna* produced 5 or 6 successive parthenogenetic generations, while *M. macrocopa* continued to reproduce without decline in fertility.

A suitable lipid-rich particle (FV) was eventually devised with albumin frac-

tion V as the fat-acceptor and coagulant. This particle served to define the need of *Moina* for fatty acids, ergocalciferol and α-tocopherol. Details on nutritional requirements are given elsewhere (Conklin and Provasoli, 1977); it suffices to say that *Moina* also needs intact nucleic acids and water soluble vitamins and that the concentrations given for the F1 medium are close to optimal under our conditions (22–24° C, subdued light). All the solids used in biphasic media are a slurry of particles ranging up to 30 μm in diameter. When added to the media, the particles remain in suspension for several hours. For maximum efficiency of ingestion the particles are resuspended twice a day by shaking the test tubes on a "Vortex Genie"; the animals are not harmed by the vigorous mixing.

Media E and F1 allow a generation time of 4–6 days, clutches of 4–8 newborn, without decline in fertility for over 200 consecutive parthenogenetic generations. The effectiveness of each variable in the diet was gauged from the number of animals produced from a single female in a week, day 1 being the day when the female produced the first brood. The variability due to the time needed for the inoculated animal (newborn or young) to produce offspring was thus avoided. In 10 ml of complete medium at the end of day 7, the *Moina* population comprised three generations: the original female, females of the first and second clutch, and their combined progeny, *i.e.*, about 13 adults and close to 100 newborn. Growth and reproduction ceased in about two weeks when almost all the particles in the 10 ml of medium were ingested.

Discussion

This report on techniques for producing several kinds of nutrient particles is motivated by the hope that other researchers may formulate better particulate media and adapt this type of media to satisfy the particulate requirements of other filter-feeders. Protozoa, sponges, rotifers, molluscs and many crustacea are filter-feeders throughout life or at least in the early larval stages; some primitive chordates such as sea squirts and salps, and some fishes are also filter-feeders.

Our experience with *Artemia* and *Moina* and the work of Akov (1962) and Dadd (1972) on mosquito larvae, indicate that success in growing filter-feeders depends on two equally important factors: supplying all the essential nutrients for growth and reproduction, and compounding the media so that the nutrients are acceptable and readily available to the animals. The biphasic media for *Moina* satisfy both requisites and result in rapid growth, high fertility, and continuous parthenogenetic reproduction.

The compromise found experimentally effective was that the nutrients which are required in large amounts by crustaceans for rapid growth must be supplied as fine particles (*e.g.*, the amino acids as precipitated proteins and the energy sources as insoluble starch and/or fats). The soluble nutrients were added at noninhibitory concentrations and high enough to compensate for the poor uptake of solutes by crustaceans. Uptake through the thick chitinous exoskeleton, except for the areas used for osmoregulation, is apparently minimal; most of the uptake is through imbibition of water while ingesting the bolus and perhaps through anal uptake (Fryer, 1970). Stephens and Schinske (1961) found that of the 11 phyla tested, only the 6 crustaceans tried were unable to take up labeled amino acids from the

environmental water. Some uptake of palmitate and glucose (at 5–250 μCi, respectively) was shown by Sargent and Lee (1975), but evidently this uptake is not sufficient to support the nutritional requirements. We found that replacements of starch and protein particles with soluble carbohydrates and amino acid mixtures was partial and inefficient: growth rates were slowed 2–3× and the solutes had 1/20–1/60 × the efficacy of particulates for *Artemia*. Therefore, crustaceans may be considered as obligate phagotrophs.

On the contrary, the work of Stephens and Schinske (1961) and later work of Stephens (1975) and Wright and Stephens (1977) shows that soft-bodied marine invertebrates are able to take up and incorporate considerable amounts of dissolved carbon sources and amino acids at the very low concentrations present in sea water. Hence, the organic solutes in biphasic media could be taken up by the soft-bodied invertebrates, and if the rate of uptake is considerable it might be necessary to lower the concentration of the present media to compensate for the increased uptake of solutes. Yet, even for these "permeable" filter-feeders the need for particulates (phagotrophy) may be postulated, because filter-feeding is a very effective gathering process as indicated by the nutritional efficiency of particles over solutes.

While most filter-feeders living in oceanic (even coastal) waters depend mostly on phagotrophy, such an assumption may not be valid for environments high in soluble organic matter (*i.e.,* high domestic pollution, where death and decay of animals or plant blooms occur, and perhaps in aerobic detrital sediments). There, organisms utilizing phagotrophy as well as osmotrophy (effective uptake of solutes) would have a great advantage: they would not depend solely on the transformation of solutes into bacteria, being able to take up solutes directly. Rasmussen (1976) has brilliantly demonstrated that the freshwater ciliate *Tetrahymena pyriformis* is almost equally efficient as an osmotroph or a phagotroph and that the uptake of solutes by *Tetrahymena* is almost as efficient as bacterial uptake. Perhaps filter-feeders in organically-rich environments employ, in different degrees, the same strategy.

Biphasic media used with germ-free techniques could be a useful tool in rearing a variety of filter-feeders and in defining their nutritional requirements. They offer a new approach because of the great experimental versatility of preparing particles of different composition and ratios and of the possibility of supplying lipids more efficiently than with emulsions which are often inhibitory.

Once nutrient requirements are understood, it may be possible to remove the limitation of germ-free handling by using microencapsulation techniques (Jones, Munford, and Gabbott, 1974). Further improvements of biphasic media under both bacterized and bacteria-free conditions should lead to the definition of the nutritional requirements of ecologically and commercially important filter-feeders. Hopefully, this data can also be applied in the formation of efficient diets for a number of anticipated aquaculture species (Provasoli, 1976).

Summary

1. Over 200 parthenogenetic generations of the freshwater Cladocera *Moina macrocopa* were obtained aseptically in three artificial media.

2. The media have two phases: a liquid phase supplying mineral salts, water

soluble vitamins, nucleic acids, and a liver extract and a fine particulate phase made from coagulated proteins, starch and lipid factors.

3. The particulate phase supplies the bulk nutrients very efficiently; hence, this type of media may be useful for growing other filter-feeders.

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